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Technical Advance

Transfection of HepG2 Cells with Infectious Hepatitis C Virus Genome

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Hepatitis C virus (HCV) represents one of the major causes of acute and chronic hepatitis, cirrhosis, and hepatocellular carcinoma (HCC) around the world. Our knowledge of the life cycle of HCV, however, is limited. Current studies are hampered by the lack of a reproducible, high-level in vitro replication system of HCV. We sought to establish HCV replication in HepG2 cells by gene transfer of *in vitro* transcribed HCV RNA. In preliminary experiments, diethylaminoethyl-dextran led to more efficient gene transfer than cationic liposomes (lipofectin, lipofectamine, and DOTAP). Therefore, in subsequent experiments, HepG2 cells were transfected with full-length (9.6-kb) and near-full-length (9.4-kb) HCV RNA using diethylaminoethyl-dextran. Transfection with subgenomic HCV RNA and mock transfection were used as controls. Positive- and negative-strand HCV RNA sequences were detected by reverse transcription polymerase chain reaction (RT-PCR) for 60 days in the infectious HCV RNA transfected HepG2 cells. The presence of negative-strand HCV RNA, presumably representing replicative intermediates, was confirmed by ribonuclease protection assay. The intracellular levels of HCV RNA were measured by quantitative competitive RT-PCR from 10 to 50 days after transfection and were stable over this time period at moderately high levels (10^8 to 10^{10} genomes per mg of total RNA). Expression of viral core and nonstructural proteins was detected in the cytoplasm of transfected cells by immunostaining. Virus-like particles measuring 50 to 60 nm in diameter were found by electron microscopy in cytoplasmic vesicles and conditioned media of the cells transfected with infectious HCV RNA but not in cells transfected with truncated HCV RNA. Culture supernatants of infectious HCV RNA transfected HepG2 cells were infectious for Daudi cells for three passages tested. The truncated HCV RNA lacking NS5 and 3′ untranslated region (3′ UTR) of HCV was replication incompetent. This is the first demonstration of HCV particles in HepG2 cells after transfection with infectious HCV RNA. We conclude that we have established a reproducible HCV replication system in HepG2 cells that can be used to study the life cycle of HCV and to test anti-HCV agents. *(Am J Pathol 1997, 151:363–373)*

Considerable progress has been made in characterizing hepatitis C virus (HCV) after the cloning and sequencing of the HCV genome in 1989.[1, 2] Recently, several laboratories have attempted to develop an HCV replication system *in vitro*. Two approaches have been used to introduce the HCV genome into cells: infection or transfection. In the infection experiments, HCV-positive plasma or serum was used to infect a variety of cells in culture, including primary chimpanzee hepatocytes,[3] Huh-7,[4] MOLT-4,[5] MT-2,[6] or B cells.[7] In these experiments, viral replication was transient or intermittent, and virus yield was low. As an alternate approach, several investigators transfected subgenomic HCV cDNA to various cell types in culture.[8–11] In these experiments, the nonstructural and structural HCV proteins were produced in various expression systems, but these constructs are not permissive for HCV replication. Full-length cDNA clones have been constructed from several positive-stranded RNA viruses and were found to be infectious in cell culture as well as in animal models.[12–16] The mechanisms by which cells produce infectious virus from a cDNA clone are not known. Presumably, the transfected DNA is transcribed in the cell nucleus to produce RNA that is transported into the cytoplasm, where it serves as mRNA to synthesize viral protein and initiate viral replication. Splicing or polyadenylation of the RNA is expected to inactivate most of the nuclear transcripts. In addition, transcription of cDNA

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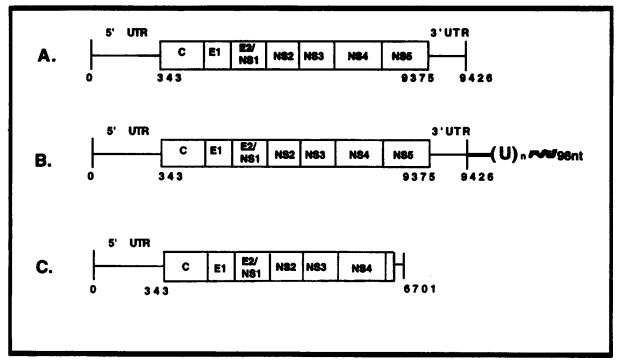
in the cell nucleus has limitations in producing biologically active viral RNA in terms of length and correct sequence at the 5′ and 3′ ends of RNA molecules. Use of *in vitro* synthesized RNA transcripts has several advantages over cDNA transfection. It avoids the involvement of the cell nucleus and potential problems regarding generation of authentic viral RNA molecules, RNA splicing, and transport to the cytoplasm. Recent studies have shown that authentic RNA transcripts, produced *in vitro* from full-length cDNA of several positive-stranded RNA viruses,[17–21] such as yellow fever virus, poliovirus, and dengue fever virus, are more infectious than cDNA clones. Yoo et al[4] have reported that *in vitro* transcribed HCV RNA is replication competent and can cause persistent infection in an hepatocellular carcinoma (HCC) cell line (Huh-7) after infectious HCV RNA transfection, but the HCV RNA titers fluctuated and the cell viability was reduced.

Recently, three groups of investigators have conclusively demonstrated the presence of a novel 98-nucleotide (nt) sequence downstream from the poly (U) stretch at the 3′ end of the HCV genome.[22–24] This novel sequence is highly conserved among different HCV isolates and genotypes. Computer modeling predicts that the 3′-terminal 98 nt form stable stem loop structures. It is likely that this conserved RNA element is involved in RNA replication, modulation of translation or RNA stability, or encapsidation of genomic RNA. On the basis of these findings, we embarked on a systematic study to establish an HCV replication system in HepG2 cells, a well differentiated hepatoblastoma cell line, by transfection with several HCV RNA transcripts.



A. 3'UTR HCV RNA 9.4
9372.....UGAGCGGGGAGCUAAACACUCCAGGCCAAUAGGCCAUCCC
CC UUUUUUUUUUUU-3'

B. 3'UTR HCV RNA 9.6
9372.....UGAGCGGGGAGCUAAACACUCCAGGCCAAUAGGCCAUCCC
CCUUUUUUUUUGUUUUUUUUUUUCUUUUUUUUUUUCUUUCCCUU
UUCCUUCUUUGGUGGCUCCAUCUUAGCCCUAGUCACGGCUAGCUG
UGAAAGGUCCGUGAGCCGCAUGACUGCAGAGAGUGCUGAUACUGG
CCUCUCUGCAGAUCAUGU-3'

Figure 1. Schematic representation of HCV RNA transcripts produced by in vitro *transcription reaction using T7 RNA polymerase* (**upper panel**) and 3′ terminal sequences of HCV RNA 9.4 kb and 9.6 kb (**lower panel**). **A:** HCV RNA 9.4 kb transcribed from plasmid construct pMO9.4-T7 with a single A at the 3′ end. **B:** HCV RNA 9.6 kb transcribed from plasmid construct pMO9.6-T7 with additional sequences at the 3′ end including poly(U) stretch, C(U) repeat, and the novel 98 nts. **C:** Truncated HCV RNA 6.7 kb transcribed from plasmid construct pMO6.7-T7 lacking most of the NS5 coding sequences and 3′ UTR, which was used as control.

Materials and Methods

Plasmid Constructs

Three plasmid constructs were prepared for *in vitro* transcription of full-length and truncated HCV RNA. Near-full-length HCV cDNA (pMO9.4) was obtained as a gift from Dr. Norio Hayashi (First Department of Medicine, Osaka University School of Medicine, Osaka, Japan).[8, 25] A PCR strategy was used to introduce a T7 promoter immediately upstream of the HCV cDNA sequence of pMO9.4. *In vitro* transcription from the T7 promoter of this construct (pMO9.4-T7) will generate near full-length HCV RNA with a single A added at the 3′ end but without the novel 98 nt. Therefore, a PCR strategy was used to insert these additional sequences[22] downstream of the HCV cDNA of pMO9.4-T7 (genotype 1b). The 3′ end sequence of the resulting construct, pMO9.6-T7, was confirmed by DNA sequencing (ds DNA Cycle Sequencing System, GIBCO BRL, Gaithersburg, MD) and is shown in Figure 1. The third construct (pMO6.7-T7) was generated by removing the *Eco*RI fragment (nt 6700 to 9426) from pMO9.4-T7. T7 transcripts from this construct lack most of the NS5 region and the 3′ UTR of HCV. The NS5b region of HCV represents an RNA-dependent RNA polymerase, and truncated forms should not replicate. This RNA was used as a control. Diagrams of the HCV RNA transcripts used in the transfection experiments are shown in Figure 1.

Preparation of HCV RNA Transcripts

HCV RNA transcripts were prepared by an *in vitro* transcription reaction using T7 or SP6 RNA polymerases (Promega, Madison, WI). Purified plasmid DNA was first linearized with *Hin*dIII restriction enzyme. HCV RNA was *in vitro* transcribed using 40 U of T7 RNA polymerase in a 40-μl reaction volume containing 40 mmol/L Tris/HCl (pH 7.5), 6 mmol/L $MgCl_2$, 10 mmol/L NaCl, 2 mmol/L spermidine, 10 mmol/L dithiothreitol, 2.5 mmol/L ribonucleotides, and 5 μg of DNA template. The reaction mixture was incubated for 1 hour at 37°C. After this, the reaction mixture was treated with RNAse-free DNAseI (RQ1 DNAse, Promega) for 1 hour and phenol/chloroform extracted. A second DNAse digestion was performed to assure no carryover of DNA in the *in vitro* transcripts. The RNA was extracted with phenol/chloroform and then with chloroform/isoamyl alcohol. Finally, the RNA transcripts were precipitated with 2.5 vol of absolute ethanol after adding 0.5 vol of 7.5 mol/L ammonium acetate. The integrity of the transcribed RNA was evaluated after electrophoresis through a 1% agarose gel.

Cell Culture and RNA Transfection

HepG2 cells were obtained from the American Type Culture Collection (Rockville, MD) and maintained in minimal essential media (GIBCO BRL) with 10% fetal bovine se-

Table 1. Nucleotide Sequences of the Oligomers Used for PCR and Southern Blot Analysis

Region	Oligomer	Oligomer sequences	Product size (bp)
HCV Core			
	First PCR		
	Sense (355)	5′CCAAAACCCCAAAGAAA3′	
	Antisense (750)	5′GTACCCCATGAGGTCGGCG3′	414
	Second PCR		
	Sense (427)	5′CAGATCGTTGGTGGAGTT3′	
	Antisense (616)	5′CAAGCCCTCATTGCCAT3′	189
	Probe (506)	5′GGTCGCAACCTCGAGGTAGACGTCAGCCT3′	
HCV NS5			
	First PCR		
	Sense (8437)	5′CGCGCGAGC GGCGTACTGACAAC-3′	555
	Antisense (8971)	5′TTGAATGATCTGAGGTAGGTC-3′	
	Second PCR		
	Sense (8451)	5′CCTGACAACTAGCTGTGGTAA3′	529
	Antisense (8959)	5′AGGTAGA TCAAGTGGTCCTAT3′	
	Probe (8584)	5′ACTCAGGAGGACGCGGCGAGCCTACGAGTCTTCACGGAGG3′	

Numbers in parenthesis denote nucleotide position.

rum, sodium pyruvate, nonessential amino acids, and antibiotics. HepG2 cells are well differentiated hepatoblastoma cells with biosynthetic capabilities similar to human hepatocytes. Separate 60-mm dishes of HepG2 cells (2×10^6 cells per dish seeded 1 day before transfection) were transfected with 20 μg of HCV RNA using the diethylaminoethyl (DEAE)-dextran method,[26] and one dish was mock transfected. For this purpose, we prepared a complex of DEAE-dextran (400 μg) and 20 μg of HCV RNA in 0.5 ml of 10 mmol/L HEPES buffer pH 7.3. After incubation for 30 minutes, 0.5 ml of complete medium was added, and the entire mixture was delivered to HepG2 cells in 60-mm dishes for 4 hours. Finally, the complex was removed, and the cells were washed three times using 5 ml of PBS and treated for 2 minutes with 2 ml of PBS containing 10% dimethylsulfoxide. The cells were then washed and maintained after adding 3 ml of complete medium.

Strand-Specific RT-PCR

Nucleic acids were extracted from transfected HepG2 cells and media at defined time points using a standard protocol and treated with DNAse I (RQ DNAse, Promega) at a ratio of 10 U/l μg of RNA. The DNA-free RNA was extracted with phenol/chloroform and precipitated with ethanol. The absence of DNA in the RNA templates was confirmed by a control polymerase chain reaction (PCR) without reverse transcriptase. RNA isolated from HepG2 cells collected immediately after HCV RNA transfection was used as a control. In all experiments, nucleic acids extracted from an HCV-positive liver specimen were included as positive controls and nucleic acids extracted from mock transfected cells and truncated HCV RNA transfected cells were used as negative controls. Amplification of HCV sequences by RT-PCR was performed as described previously.[27] The sequences of the primers and probes used in the RT-PCR and Southern blots, respectively, are shown in Table 1. To demonstrate production of full-length HCV RNA in the transfected HepG2 cells, we used two sets of PCR primers located within the 5′ and 3′ UTRs of the HCV genome. One microgram of RNA extracts prepared from cells or 100 μl of spent medium was used along with 250 ng of outer antisense primer for cDNA synthesis for the detection of positive-strand HCV RNA. For reverse transcription (RT), 10 U of avian myeloblastosis virus reverse transcriptase (AMV RT) enzyme was added and cDNA synthesis performed for 1 hour at 42°C. The resulting cDNA was amplified by PCR after adding the outer sense (OS) primer (250 ng) to 50 μl of PCR reaction mixture containing 2.5 U of *Taq* DNA polymerase. A second PCR was carried out with a set of inner primers (250 ng each) using 5 μl of the first PCR product as DNA template. The cDNA synthesis from the negative-strand HCV RNA was performed using the OS primer for NS5 or core sequences under the same conditions as described above. After reverse transcription, AMV RT was inactivated by heating the reaction mixture in a boiling water bath for 60 minutes. Four different controls (no RNA, no RT enzyme, no OS primer in the RT reaction, and no antisense primer in PCR) were included to demonstrate the specificity of the RT-PCR for negative-strand HCV RNA as described by Fong et al[28] and Landford et al.[3] The amplification products were electrophoresed on 2% agarose gels, and the specificity of the amplicons was confirmed after Southern blotting using the appropriate HCV probe (Table 1).

The following experiments were performed to demonstrate the sensitivity and specificity of our RT-PCR assay. For this purpose, *in vitro* transcribed positive- and negative-strand HCV RNAs were prepared as described above, and plasmid DNA templates were eliminated by repeated DNAse I (RQ1 DNAse, Promega) digestion. Five micrograms of *in vitro* transcribed HCV RNA was diluted with HepG2 cell RNA (5 μg) to mimic the conditions for detection of HCV in the transfected cells and digested with DNAse (10 U/1 μg) for 1 hour at 37°C. Then, 300 μl of guanidinium isothiocyonate (GITC) solution was added to the RNA, extracted with phenol/chloroform, and precipitated with ethanol. Positive- or negative-strand HCV RNAs were reverse transcribed with antisense and sense HCV core primers, respectively, and

RNA templates and RT enzyme were destroyed by boiling in a water bath for 1 hour. The HCV cDNA was amplified by nested PCR after adding the appropriate primers as described above. The sensitivity of the RT-PCR assay was determined by serially diluting positive- and negative-strand synthetic HCV RNA templates. The titration of RT-PCR for positive- and negative-strand HCV RNA after Southern blotting is shown in Figure 3A. In two experiments, less than 10 HCV RNA copies per assay was detected. The level of strand specificity was determined using 1 pg of HCV RNA employed in the titration experiments. This amount is equivalent to approximately 10^5 HCV RNA molecules.

Ribonuclease Protection Assay

A second method for demonstration of negative-strand HCV RNA in the transfected HepG2 cells was employed. A 296-nt sense riboprobe for the 5′ UTR of HCV RNA was transcribed from a *Hin*dIII-digested plasmid construct (PCR II-296 containing nt 46 to 341 of HCV) by T7 RNA polymerase in the presence of [^{32}P]UTP. The probe length was 422 nt, with 126 nt derived from the plasmid vector PCR II (Invitrogen, San Diego, CA). The 422-nt riboprobe was gel purified and hybridized to 50 μg of total RNA isolated from the HCV transfected HepG2 cells in 20 μl of hybridization buffer. The samples were denaturated at 85°C for 5 minutes and hybridized overnight at 42°C. RNAse digestion was performed using a commercially available ribonuclease protection assay kit (Ambion, Austin, TX). Finally, the protected fragments were separated on 8 mol/L urea/6% polyacrylamide gels and visualized by autoradiography. The size of the protected fragment was compared with that of a positive control. *In vitro* transcribed positive- and negative-strand HCV RNA (10 μg) was used to demonstrate the specificity of the assay.

Quantitation of HCV RNA Level by Competitive RT-PCR

Competitive RT-PCR is based on the principle of co-amplification of the target RNA with known amounts of synthetic mutated RNA as internal standard. Dr. Norio Hayashi (Osaka University School of Medicine, Osaka, Japan) generously provided the mutated HCV cDNA in pBluescript II vector with a new *Eco*RI site in the 5′ UTR generated by site-directed mutagenesis. The same competitive RT-PCR method as described by Hagiwara et al[29] was used. The assay was calibrated with a standard HCV-positive serum (N105 with a chimpanzee infectivity titer of $10^{6.5}$ Chimpanzee Infectious Dose (CID) per ml and 10^7 HCV genomes per ml) and had a limit of detection of 10^3 HCV genomes per ml.

Immunocytochemical Detection of HCV Proteins

Immunostaining for HCV protein (core, NS3, NS4, and NS5) was performed on the HepG2 cells at 30 and 50 days after transfection as described previously.[30] Briefly, transfected and control cells were grown on chamber slides, fixed in cold acetone, washed, and incubated overnight with polyclonal rabbit anti-HCV antibodies (generously provided by Dr. Johnson Lau, University of Florida, Gainesville, FL), followed by biotinylated anti-rabbit antibody for 30 minutes. Avidin-biotin-peroxidase complex (Vectastain Elite ABC kit, Vector Laboratories, Burlingame, CA) was added, followed by diaminobenzidine/hydrogen peroxide substrate and counterstaining with hematoxylin. Rabbit IgG and PBS were used as controls.

Demonstration of HCV Particles by Electron Microscopy

The HCV RNA transfected HepG2 cells were examined at 3 days after transfection for the presence of virus-like particles by electron microscopy. Pellets of the transfected and control HepG2 cells were fixed in 3% glutaraldehyde, postfixed in 1% osmium tetroxide, dehydrated in ethanol, infiltrated with propylene oxide, and embedded in Epon mixture. Ultrathin sections were stained with a saturated solution of uranyl acetate and lead citrate and examined by transmission electron microscopy using a Hitachi H.7100 electron microscope without knowledge of the origin of the cells.

For immunoelectron microscopy, the medium from the transfected cells at 7 days was cleared by low-speed centrifugation and centrifuged at 75,000 × *g* for 6 hours at 4°C as described by Kato et al.[6] The pellet was resuspended in 100 μl of PBS and incubated for 2 hours at 4°C with 2 μl of monoclonal anti-envelope antibody (anti-E1, generously provided by Dr. Johnson Lau, University of Florida). The mixture was centrifuged again at 75,000 × *g* for 2.5 hours at 4°C, and the pellet was dissolved in 100 μl of PBS, placed on Formvar-coated grids, and examined under the electron microscope after negative staining with 1% phosphotungstic acid.

Infectivity Assay

Culture supernatants collected from the transfected HepG2 cells were tested for the presence of infectious virus. For this purpose, the infectivity assay reported by Shimizu et al[31] was employed using media collected 30 and 40 days after transfection. Daudi cells (5×10^5 cells) were incubated with 1 ml of conditioned culture medium for 4 hours at 37°C. Conditioned medium from HepG2 cells transfected with truncated HCV RNA 6.7 was used as control. The cells were washed twice and suspended in 3 ml of fresh medium (RPMI with 20% fetal bovine serum). Virus replication in Daudi cells was determined by detecting intracellular negative-strand HCV core RNA by RT-PCR at 2 weeks after infection. The infectivity assay was performed for two additional cycles using conditioned media of Daudi cells 3 and 2 weeks after infection, respectively (see Figure 10).

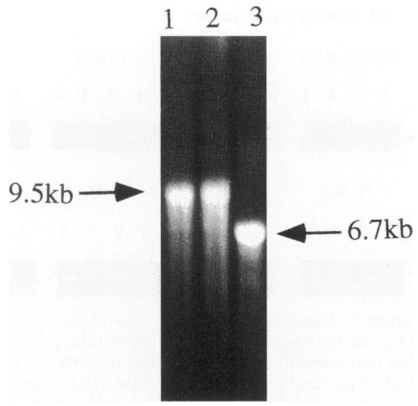


Figure 2. In vitro *transcribed genomic and subgenomic HCV RNAs used for transfection of HepG2 cells. Approximately 1 μg of HCV RNA was electrophoresed in a 1% agarose gel and stained with ethidium bromide.* **Lane 1**, HCV RNA 9.4 kb; **lane 2**, HCV RNA 9.6 kb; **lane 3**, HCV RNA 6.7 kb.

Results

HCV RNA Transcripts

Three plasmid constructs (Figure 1) were used to transcribe full-length genomic and subgenomic HCV RNA. The transcripts were synthesized *in vitro* by T7 RNA polymerase from linearized plasmid DNA. The *in vitro* transcribed RNA was treated with DNAse I, phenol/chloroform extracted, and precipitated with ethanol. The size of *in vitro* synthesized RNA was determined by electrophoresis on a 1% formaldehyde agarose gel followed by Northern blot hybridization analysis and revealed that >90% of the transcribed RNA was of the expected (9.6-, 9.4-, and 6.7-kb) sizes (Figure 2). The presence of 3′ terminal sequences in the *in vitro* transcripts of 9.6-kb HCV RNA was confirmed by Northern blotting using an oligo-probe located within the 98 nt. The *in vitro* transcription products were digested by DNAse I or RNAse A to ensure that the transcription product was RNA. Before each HCV RNA transfection, the integrity of *in vitro* transcribed RNA was again evaluated on a 1% agarose gel.

Six different methods were employed to evaluate *in vitro* production of HCV in HepG2 cells after transfection at defined time points.

Strand-Specific RT-PCR

HCV core region sequences were detected by RT-PCR in the cells and medium of HepG2 cells transfected with 9.6- and 9.4-kb HCV RNAs but not in mock transfected HepG2 cells. HCV RNA was found in HepG2 cells transfected with the truncated form (6.7 kb) of HCV RNA immediately after transfection but not at later time points (Table 2). In initial experiments, a branched DNA assay (Quantiplex version 2.0, Chiron Corp., Emoryville, CA) demonstrated that the levels of input RNA for all clones, including the 6.7-kb HCV RNA, were similar immediately after transfection ($>9.5 \times 10^6$ copies/1 mg of total RNA). The presence of viral RNA was demonstrated in 100 μl of conditioned medium using RT-PCR for NS5 at 0, 3, 10, 20, 30, 40, 50, and 60 days after transfection. The control media and reagents tested at the same time were negative.

HCV is a positive-strand RNA virus and presumably replicates via a negative strand. Therefore, virus replication was investigated by strand-specific RT-PCR for negative-strand sequences. Four different controls (no RNA, no RT enzyme, no OS primer in the RT reaction, and no antisense primer in the PCR) were included routinely as specificity controls for the negative-strand RT-PCR and were always negative. The controls described above and alternate detection methods are crucial because several reports have been published suggesting that the standard RT-PCR method for detection of negative-strand HCV RNA lacks strand specificity. This may be due to reverse transcriptase activity of *Taq* polymerase or due to false priming, self-priming, or random priming of the wrong strand during the RT step. Strand specificity of the RT-PCR assay was tested using 1 pg of HCV RNA that was premixed with an equal amount of HepG2 cell RNA (Figure 3B). No positive-strand HCV RNA was detected in the RT-PCR for negative-strand HCV RNA and no negative-strand HCV RNA was detected in the RT-PCR for positive-strand HCV RNA. The level of sensitivity of the RT-PCR procedures for both positive and negative strands was less than 10 HCV RNA copies per assay as shown by serial dilution (Figure 3A). As an additional control, we tested the presence of negative-strand HCV RNA by RT-PCR using core primers in the cells transfected with truncated 6.7-kb HCV RNA, and the results were negative. In contrast, intracellular negative-strand HCV RNA was detected 3 to 60 days after transfection with the 9.6- and 9.4-kb HCV RNA constructs (Figure 4; Table 2). No negative-strand HCV RNA was detected in the cells immediately after transfection. HepG2 cells transfected with either of these constructs exhibited cytotoxic effects, and cell density decreased over this time

Table 2. RT-PCR Results of HCV RNA Transfection of HepG2 Cells

Days after transfection	HCV RNA 9.4-kb transfected cells HCV core RNA sequences		HCV RNA 9.6-kb transfected cells HCV core RNA sequences	
	Positive strand	Negative strand	Positive strand	Negative strand
0	−	−	−	−
3	+	+	+	+
30	+	+	+	+
50	+	+	+	+
60	+	+	+	+

Positive strand HCV RNA was also tested by RT-PCR at days 10, 20, 30, and 40 after transfection and was positive (+). Truncated HCV RNA transfected cells (except immediately after transfection) and mock transfected cells were always negative (−).

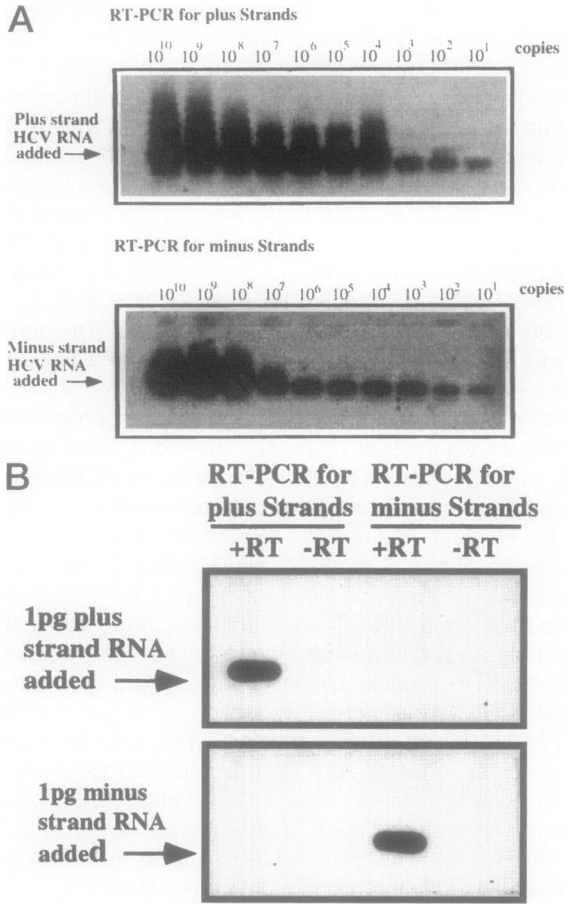


Figure 3.A: Sensitivity of RT-PCR assays for positive and negative strand HCV RNA using a set of core primers. Positive- and negative-strand HCV RNA transcripts were prepared as described in the text, and plasmid DNA templates were eliminated with repeated DNAse digestion. Serial dilutions of the synthetic RNAs were tested by RT-PCR for core sequences. The results of RT-PCR were confirmed after Southern blotting. The **upper panel** shows the sensitivity of RT-PCR for positive-strand RNA using 10-fold serial dilutions. The **bottom panel** shows the sensitivity of RT-PCR for negative-strand RNA using 10-fold serial dilutions. The assay detected less than 10 copies of HCV RNA. **B:** Strand specificity of RT-PCR for HCV RNA using core primers. Positive- and negative-strand HCV RNAs were *in vitro* transcribed by T7 polymerase and SP6 RNA polymerase, respectively. The plasmid DNA templates were eliminated by repeated DNAse I digestion. Dilutions of synthetic RNAs were made in total cellular RNA to mimic the conditions for analysis of transfected cells. For RT-PCR, 1 pg of positive- or negative-strand HCV RNA was subjected to reverse transcription with antisense and sense primers, respectively. The samples were then boiled for 1 hour to eliminate the reverse transcriptase and RNA template, and first round PCR amplification was carried out after adding the second primer. Nested PCR was performed using 5 μl of first PCR products as template, and 10 μl of nested PCR products was analyzed by agarose gel electrophoresis and Southern blot hybridization. The **upper panel** shows no detectable negative-strand HCV RNA in the in vitro *transcribed positive-strand RNA templates. The* **bottom panel** shows no detectable positive-strand RNA in the *in vitro* transcribed negative-strand RNA templates. ± RT, with or without reverse transcriptase, respectively.

period. Further detection of viral RNA was not possible as the cells died between 50 and 60 days after transfection. In contrast, cells transfected with truncated HCV RNA or mock transfected showed no toxicity and became confluent at 3 days.

Ribonuclease Protection Assay

As an alternative method to demonstrate negative-strand HCV RNA sequences in transfected HepG2 cells, we

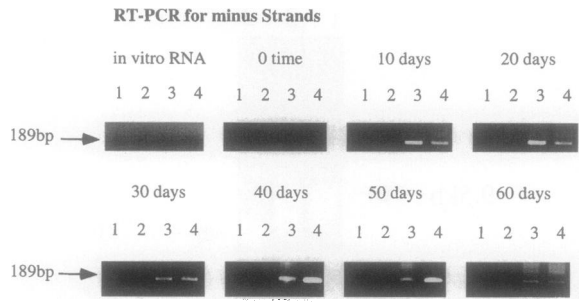


Figure 4. Ethidium bromide staining of RT-PCR products for negative-strand HCV RNA in transfected HepG2 cells at different time points. For in vitro *RNA, 5 μg of in vitro* transcribed positive-strand HCV RNA was diluted with HepG2 cell RNA (5 μg) and digested with DNAse (10 U/1 μg) for 1 hour at 37°C. Then, 300 μl of GITC solution was added to the RNA, extracted with phenol-chloroform, and precipitated with ethanol. Strand-specific RT-PCR for negative-strand HCV RNA was performed using 200 ng of RNA. Negative-strand HCV RNAs were reverse transcribed with sense HCV core primers for 1 hour at 42°C. After this step, the RNA templates and RT enzyme were destroyed by boiling in a water bath for 1 hour. The HCV cDNA was amplified by nested PCR after adding the appropriate primers. **Lane 1**, reagent control; **lane 2**, HCV RNA 6.7; **lane 3**, HCV RNA 9.4; **lane 4**, HCV RNA 9.6. Shown at time 0 is the absence of negative-strand HCV RNA in the HepG2 RNA extracts, which were collected immediately after transfection. Also shown are the negative strands in the HepG2 cells at 10, 20, 30, 40, 50, and 60 days after transfection. **Lane 1**, mock transfected HepG2 cells; **lane 2**, HepG2 cells transfected with truncated HCV RNA 6.7; **lane 3**, HepG2 cells transfected with HCV RNA 9.4; **lane 4**, HepG2 cells transfected with HCV RNA 9.6. The 189-bp negative-strand amplification product was seen only in cells transfected with HCV RNA 9.4 and 9.6. The specificity of the bands was confirmed by Southern blotting.

employed the ribonuclease protection assay, using a sense riboprobe corresponding to nt sequences 46 to 341 of the 5′ UTR of the HCV genome. Synthetic positive- and negative-strand HCV RNAs, HepG2 cells transfected with truncated HCV RNA, and mock transfected cells were used as controls. As shown in Figure 5, we observed the expected 296-nt protected fragment in the HepG2 cells at 7 days after transfection with infectious HCV RNAs but not in the controls. Attempts to demonstrate negative-strand HCV RNA by Northern blot were not successful.

Quantitation by Competitive RT-PCR

The amount of viral RNA in the transfected cells was quantitated by competitive RT-PCR at five time points after transfection with 9.6- and 9.4-kb HCV RNA. The intracellular HCV RNA levels were fairly stable from 10 to 50 days after transfection ranging from 10^8 to 10^{10} HCV RNA copies per mg of total cellular RNA (Figure 6). The earliest time point selected for the quantitative assay was 10 days to avoid measuring HCV RNA carryover from the inoculum. The assay was performed twice by different investigators and yielded identical results. We did not detect a significant difference in HCV RNA levels in HepG2 cells transfected with the 9.6- or 9.4-kb HCV RNA. This level was similar to the amount of HCV RNA found in infected human liver (10^5 to 10^{10} molecules/mg of liver RNA) as reported previously.[35]

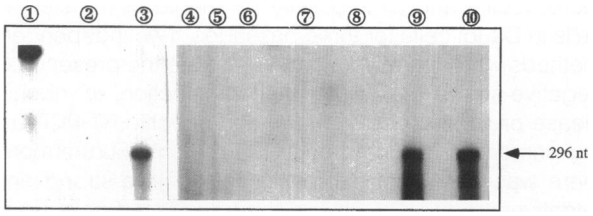


Figure 5. RNAse protection assay for detection of negative-strand HCV RNA in transfected HepG2 cells and controls. RNA extracted from transfected cells was probed with a ^{32}P-labeled, gel-purified, positive-strand RNA probe for the 5′ UTR. The RNA samples were denatured and hybridized overnight at 42°C. RNAse digestion was performed using an RNAse protection assay kit (Ambion). The products were resolved on a 6% polyacrylamide gel containing 8 mol/L urea and exposed to Kodak X-Omat AR film. **Lane 1**, probe alone; **lane 2**, RNAse-digested probe; **lane 3**, *in vitro* transcribed negative-strand HCV RNA used as a positive control showing a 296-nt protected fragment; **lanes 4** to **6**, 10 μg of *in vitro* transcribed positive-strand HCV RNA (HCV RNA 6.7, HCV RNA 9.4, and HCV RNA 9.6 respectively); **lane 7**, mock transfected HepG2 cells; **lane 8**, HepG2 cells transfected with truncated HCV RNA 6.7; **lane 9**, HepG2 cells transfected with HCV RNA 9.4; **lane 10**, HepG2 cells transfected with HCV RNA 9.6. A 296-nt protected fragment is present in cells transfected with HCV RNA 9.4. and 9.6 but not in the controls. The film was exposed up to 6 days, and no signal was seen in the control lanes.

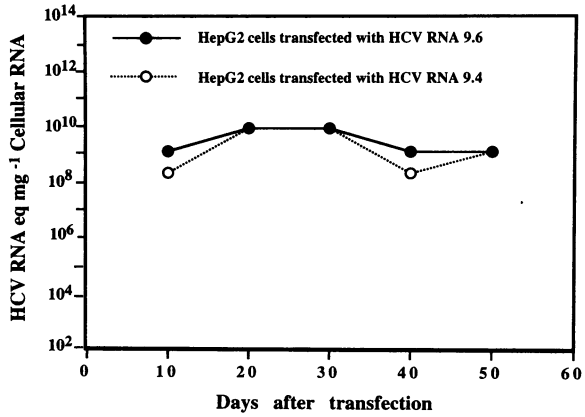


Figure 6. Amount of HCV RNA in transfected HepG2 cells over the course of 50 days as determined by quantitative competitive RT-PCR for the 5′ UTR region. The levels of HCV RNA in the transfected cells were fairly constant (10^8 to 10^{10} HCV RNA genomes per mg of total cellular RNA).

Immunohistochemical Demonstration of HCV Proteins

Expression of HCV proteins (core, NS3, NS4, and NS5) was observed as a finely granular reaction product in the cytoplasm of 30 to 60% of cells tested at days 30 and 50 after transfection, whereas staining with a control antibody was negative (Figure 7). The control cells (mock transfected and transfected with 6.7-kb HCV RNA) were negative after staining with the same polyclonal anti-HCV antibodies.

Electron Microscopy

HepG2 cells were examined at 3 days after transfection by transmission electron microscopy. Virus-like particles measuring 50 to 60 nm in diameter were found in cytoplasmic vesicles in rare cells after transfection with 9.6- and 9.4-kb HCV RNA but not in mock transfected cells (Figure 8, A and B). The morphology and predicted size of the particles were consistent with previous reports.[32, 33] Immunoelectron microscopy of conditioned medium from cells 7 days after transfection was performed using monoclonal antibodies to HCV envelope. As shown in Figure 9, small aggregates of 50 to 60-nm enveloped particles were detected in the culture supernatants of cells transfected with 9.6- and 9.4-kb HCV RNA but not with 6.7-kb HCV RNA.

Infectivity Assay

Using the infectivity assay described by Shimizu et al,[30] we incubated Daudi cells for 4 hours with conditioned medium collected from HepG2 cells 30 and 40 days after transfection with 9.6- and 9.4-kb HCV RNA. Negative-strand HCV RNA was detected in the Daudi cell pellet at 2 weeks after infection. Two additional cycles of infection of naive Daudi cells with supernatant medium from infected Daudi cells also demonstrated negative-strand HCV RNA (Figure 10). These findings suggest that the culture supernatants collected from the transfected HepG2 cells were infectious for Daudi cells. Medium from truncated HCV RNA transfected HepG2 cells was used as control and yielded negative results.

Discussion

Significant progress has been made in several laboratories in the development of an *in vitro* cell culture system of HCV. Two types of cultured cells were used: liver-derived cells such as primary chimpanzee hepatocytes[3] or a well differentiated human HCC cell line[4] and lymphoid cell lines of either B-cell[7] or T-cell[5, 6] origin. In a series of publications,[6, 31–33] Purcell's group demonstrated that human T-cell lines can be infected by HCV. A clone of the HPB-ALL human T cells was used to develop an *in vitro* infectivity assay for HCV.[30] Replication and expression of HCV were evaluated by detection of intracellular negative-strand RNA, which serves as a template for the synthesis of virion RNA and by indirect immunofluorescent staining for the HCV core antigen. The intracellular expression of HCV RNA, however, fluctuated; quantitative assays were not employed to demonstrate the level of viral replication; and the efficiency of infection appeared to be low. Recently, Shimizu et al[32] demonstrated the detection of intracellular HCV particles by electron microscopy in Daudi cells, an Epstein-Barr-virus-transformed B-lymphoblast cell line, after infection with HCV-positive plasma. This represented the first convincing visualization of complete HCV virions in cultured cells and was supported by immunoelectron microscopy with antibody to the HCV envelope protein E2. Approximately 20% of cells were positive by immunofluorescence staining for core and envelope antigens. These studies suggested that infected Daudi cells produced infectious HCV for more than 6 months.

Yoo et al[4] employed a different approach. They used T7 RNA polymerase transcripts of a near-full-length cDNA clone of HCV to transfect a differentiated human

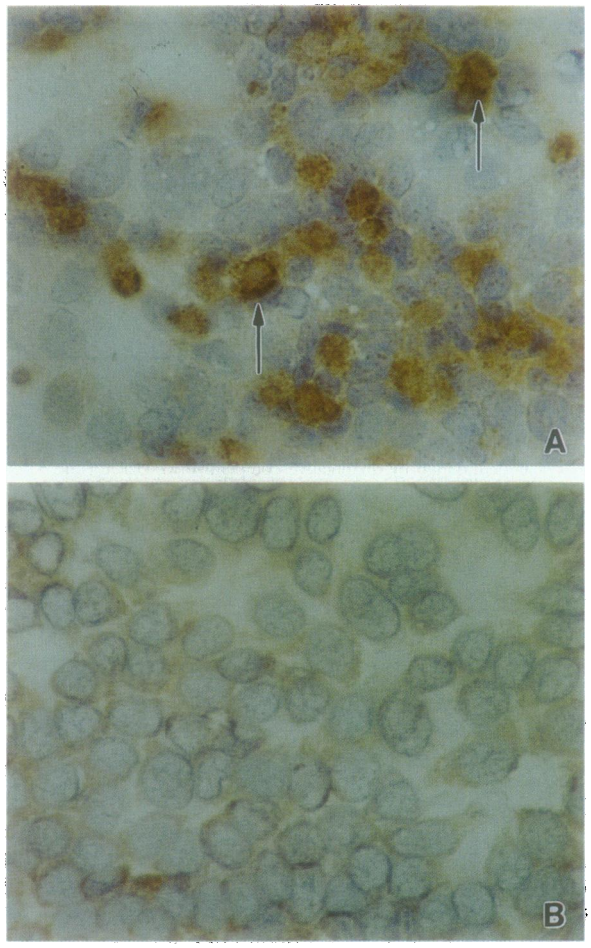


Figure 7. Immunohistochemical staining for HCV core protein in the transfected HepG2 cells at day 30 after transfection. Granular expression of HCV core protein is seen in the cytoplasm (**arrows**) of 35% of the transfected HepG2 cells (**A**), whereas mock transfected HepG2 cells are negative (**B**). Original magnification, ×400.

HCC cell line, Huh-7. The transfected genome appeared to replicate in the cells as evidenced by the finding of progeny HCV RNA, detection of negative strands and incorporation of [^{3}H]uridine into the viral genome. The conditioned medium of transfected Huh-7 cells was infectious for naive Huh-7 cells, suggesting the production of biologically active virus. Again, however, HCV replication fluctuated and HCV titers diminished over a period of 4 months. The infected cells formed aggregates, and their viability was considerably reduced. This represented the first demonstration that HCV RNA produced from cloned HCV cDNA was infectious and replication competent.

In this study, we investigated HCV replication in HepG2 cells after transfection with full-length (9.6-kb) and near-full-length (9.4-kb) HCV RNA transcripts. We believe that we demonstrated viral replication in transfected HepG2 cells by several lines of evidence: 1) detection of negative-strand HCV RNA, 2) stable intracellular HCV RNA levels over a time period of 60 days, 3) demonstration of HCV-like particles in transfected cells and their media, 4) expression of viral proteins in transfected cells, and 5) infectivity by media of transfected cells in Daudi cells for three passages. Two independent methods were employed to demonstrate the presence of negative-strand HCV RNA after transfection, ie, ribonuclease protection assay and strand-specific RT-PCR using primers for the core and NS5 regions. Furthermore, there was persistent expression of positive-strand and negative-strand HCV RNA in the transfected cells up to 60 days after transfection by RT-PCR. A previous report[34] demonstrated the presence of a small amount of complementary RNA in *in vitro* transcripts made with T7 RNA polymerase. We did not detect complementary RNA in our *in vitro* transcribed positive-strand HCV RNAs based on the following evidence: 1) our input RNA was devoid of negative-strand HCV RNA by strand-specific RT-PCR, 2) we did not detect any negative-strand HCV RNA in the HepG2 cells immediately after transfection, and 3) the *in vitro* transcribed ^{32}P-labeled RNA probe was completely digested with a mixture of RNAse A and T1 (as shown by the ribonuclease protection assay in Figure 5). The cells transfected with 9.6- and 9.4-kb HCV RNA showed significant cytotoxicity, and cell density decreased over a

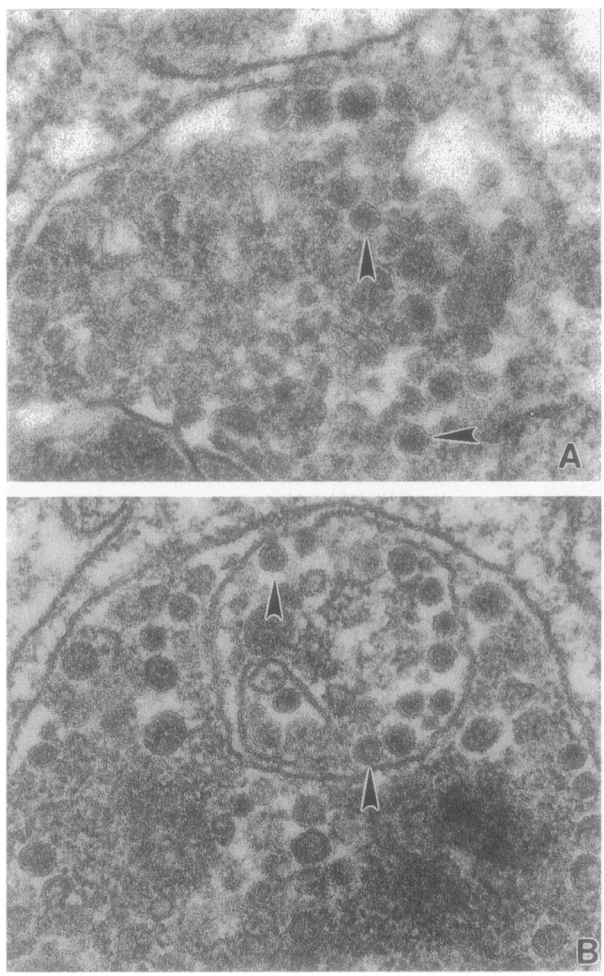


Figure 8. Electron micrographs of HCV particles in HepG2 cells 3 days after transfection with HCV RNA. Virus-like particles measuring 50 to 60 nm in diameter (**arrows**) are seen in cytoplasmic vesicles of cells that were transfected with HCV RNA 9.4 (**A**) or HCV RNA 9.6 (**B**) but not in mock transfected cells. Original magnification, ×65,000 (**A**) and ×60,000 (**B**).

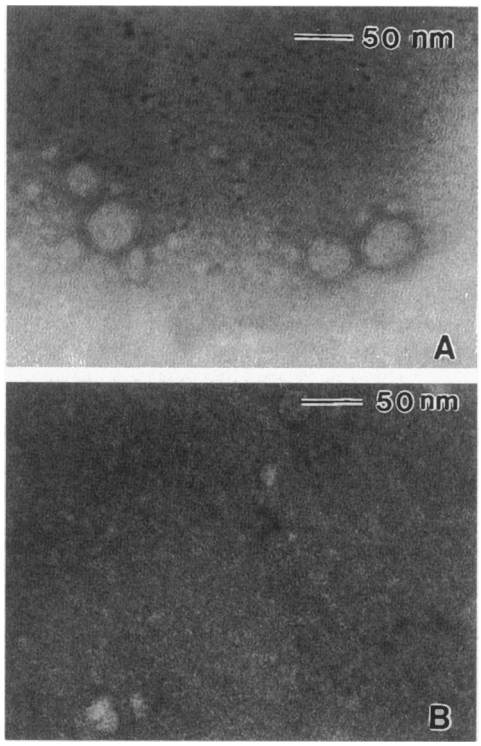


Figure 9. Immunoelectron micrographs of HCV particles from the medium of transfected HepG2 cells using a monoclonal anti-envelope antibody and negative staining with 1% phosphotungstic acid (PTA). Aggregates of virus-like particles measuring 50 to 60 nm in diameter are seen in the media of HepG2 cells transfected with HCV RNA 9.6 (**A**) but not in media of cells transfected with HCV RNA 6.7 (**B**).

2-month period. The toxicity was not due to incubation with DEAE-dextran, because the mock transfected HepG2 cells and HepG2 cells transfected with truncated HCV RNA became confluent 3 days after transfection. Studies are underway to determine the effect of virus replication on cell function and viability.

To exclude the possibility of HCV RNA carryover from the original inoculum, we studied multiplication of HCV in the cultures by quantitative competitive RT-PCR. As shown in Figure 6, the amount of HCV RNA in transfected cells remained fairly stable between 10 and 50 days after transfection with the 9.6- or 9.4-kb HCV RNA transcripts. The amount of HCV RNA (10^8 to 10^{10} HCV genomes per mg of total cellular RNA) was similar to that in a recent study by McGuinness et al,[35] who reported HCV RNA levels in infected human liver from 10^5 to 10^{10} per mg of total liver RNA using dot-blot PCR. In an initial experiment (not shown), the amount of HCV RNA in pooled cells and media increased from 1.15×10^7 to 4.4×10^7 genome equivalents per mg of total RNA between 30 and 50 days after transfection with 9.4-kb HCV RNA as determined by branched DNA assay. Taken together, these results suggest multiplication of virus in the cultures and exclude the possibility of carryover of the input RNA.

Recent reports by Tanaka et al[22] and Kolykhalov et al[23] demonstrated the presence of an additional 98-nt stretch at the 3′ end of HCV. The sequence is highly conserved and is likely to play a critical role in HCV replication. Yet, in our hands, not only the 9.6-kb HCV RNA clone with the 98-nt stretch but also the 9.4-kb HCV clone without the 98-nt sequence was infectious. This finding is supported by several published reports. First, Yoo et al[4] reported that HCV genotype 1, which ended with a 15-mer of A (1a) or U (1b) bases downstream of the conventional 3′ UTR, was infectious for Huh-7 cells. Second, Behrens et al[36] demonstrated that HCV RNA-dependent RNA polymerase can initiate synthesis of negative strands from a 3′ UTR of HCV RNA 9.4. Third, our 9.4-kb HCV RNA has 54 nt at the 3′ UTR.[8, 25] Molecular modeling of this short UTR sequence was shown to form a primary stem loop structure.[37] This suggests that the 54 nt present in the 9.4-kb HCV RNA are important in the replication of the viral genome. Fourth, studies of other flaviviruses, such as tick-borne encephalitis virus[38] and dengue type 4 virus,[39] indicate that virus replication can be seen even when there is a variation in the length of the 3′ UTR. The sequence of the 3′ UTR of our 9.6-kb HCV RNA construct consists of a type-specific region (after the termination codon), a poly(U) stretch, a C(U) repeat, and the novel 98-nt stretch (shown in Figure 1). The sequences are identical to those described for genotype 1b,[22] except for the length of poly(U). The 9.6-kb construct used in our study contains a poly(U) stretch of 32 nt. Recent reports[24, 40] suggest that the length of the poly(U) stretches varies (20 to 60 nt) among the genotypes of HCV and did not affect the secondary structure of the 98-nt stretch. We are currently studying whether the level or persistence of replication after transfection with full-length *versus* the near-full-length HCV RNA transcripts are different. Future studies will address the importance of the primary and

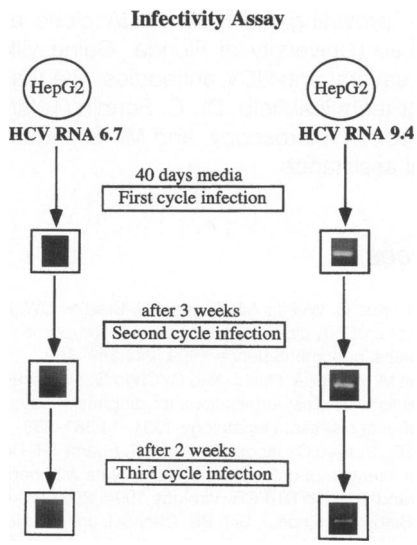


Figure 10. Infectivity assay of the conditioned media from transfected HepG2 cells (at day 40) to Daudi cells. The presence of HCV replication in the infected Daudi cells was detected by RT-PCR for intracellular negative-strand HCV core sequences. A Daudi cell suspension (0.5×10^6 cells) was mixed with 1 ml of cell-free conditioned medium and incubated for 4 hours at 37°C. Cells were then washed and incubated with fresh medium. Cells were maintained by changing the medium every 3 days. The medium of the infected Daudi cells was used for two more passages, 3 and 2 weeks after infection, respectively. Two weeks after infection, HCV replication in the infected cells was detected by strand-specific RT-PCR for intracellular negative-strand HCV core sequences.

secondary stem loop structures in replication and packaging of complete virus particles.

We demonstrated by *in vitro* infectivity assays that HepG2 cells transfected with 9.6- or 9.4-kb HCV RNA transcripts produced infectious HCV over the course of three passages. Virus-like particles measuring 50 to 60 nm in diameter were found in HepG2 cells transfected with both constructs. The size and morphology of the particles were similar to those previously reported by Shimizu et al[32] and Kaito et al[41] and suggested the production of complete viral particles from HCV RNA transcripts.

The development of a high-level replication system for HCV reported here differs in several significant aspects from previous attempts. We used full-length and near-full-length HCV RNA transcripts of genotype 1b, which has been clinically associated with higher virus titers and more severe disease than other genotypes. HepG2 cells, a well differentiated human hepatoblastoma cell line that maintains many liver-specific functions in culture, was transfected by an efficient gene transfer method as determined in extensive preliminary experiments. The quality and purity of HCV RNA transcripts used for transfection was carefully monitored. *In vitro* production of HCV in HepG2 cells for 2 months was demonstrated by several independent methods. Therefore, we believe that we have established an *in vitro* HCV replication system that will be useful in future studies of the life cycle of HCV and the development of anti-HCV agents.

Acknowledgments

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